ACTIVE IMMUNIZATION VS COCCIDIOIDES IMMITIS

A Summary Report on Research from March 1, 1965 to July 31, 1965

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SEROLOGICAL DATA

A. General Aims and Approach:

1. Cellular, sonicated and autoclaved-sonicated antigens derived from arthrospores and from spherules were tested by the complement fixation method against cooled sera obtained from rabbits infected with C. immitis. These tests were performed to detect antigenic differences between arthrospores and spherules. An attempt also was made to correlate these results with previous data obtained by testing similar antigens with immune rabbit sera.

An agar-gel double diffusion assay was performed to demonstrate the number of precipitin bands present in soluble extracts derived from arthrospores and from spherules, and the relationship of these precipitin bands among the extracts.

The indirect immuno-fluorescent assay was utilized to confirm the antigenicity of the cell wall of arthrospores and of spherules, and of their articulate fractions.

B. Methods and Materials:

1. Complement fixation: The Kolmer one-fifth volume complement fixation test, as outlined in the previous tri-annual report, was the assay used to test the pooled sera from infected rabbits.

The cellular, sonicated, and autoclaved-sonicated antigens were prepared according to the methods outlined in the previous tri-annual report.

Three white rabbits were injected with C. immitis in the following manner. The growth from four Sabouraud's agar slants of C. immitis, strain M 11-10 was washed from the slants with sterile physiological saline. The resulting mixture was poured into a sterile tissue grinder and subjected to fifteen complete cycles

strokes. This procedure removed all large clumps and produced a homogenous suspension. The mixture was then placed in a sterile conical centrifuge tube, and centrifuged for ten minutes at 2000 X G in an international centrifuge; this sediment was used to prepare a 10% suspension in sterile saline. A ten fold dilution was then made of this stock suspension. After preinfection sera was drawn from the marginal ear vein of three rabbits, 1.0 ml of this ten fold dilution was injected, i.v., into each animal. Colony counts of the rabbit inoculum on Sabouraud's antibiotic agar revealed approximately 50,000 infectious particles per ml.

Twenty-five days after infection, the animals were bled out by cardiac puncture, and the sera obtained from the two animals showing the highest complement fixation titers were pooled and frozen.

Autopsies performed on the exsanguinated animals revealed the lungs, liver, spleen, and kidneys to be covered with a small caseous lesion. Microscopic preparations of the affected tissues revealed typical spherules of C. immitis, and cultures of the liver, spleen, and lungs made on Sabouraud's antibiotic agar were positive for C. immitis.

2. Agar-gel Double Diffusion. The agar-gel double diffusion technique used in this study was a modification of the technique of Ouchterloney (1949). The agar used in the gel diffusion plates was 0.4 per cent Ionagar (Oxoid) which was dissolved in physiological saline with 1-10,000 merthiolate v/v added. Thirteen mls. of this agar was poured into sterile plastic petri dishes and allowed to harden.

Two different agar-gel double diffusion patterns were used in this study. In the first pattern six outer wells were cut

with a #3 cork borer, each 9 mm from a center well which was cut with a #4 cork borer. The outer wells were cut in a hexagonal pattern. A second pattern was made by surrounding a center hole, cut with a #4 cork borer, with four outer wells, cut with a #3 cork borer. In these plates the outer wells made a square pattern. The center well was located 9 mm. from the outer wells, with the exception of the plates shown in Figures 9 and 10. In these plates the center well was situated 7 mm from the outer ones. After the patterns were cut, the excess agar was removed by aspiration.

When the hexagonal pattern was used, the various antisera were added to the outer wells, and the plates incubated at 37°C for two hours. The antigen was then added to the center cavity, and the plates were incubated at 4 - 8° C for seven to ten days. When the square pattern was used, the same procedure was followed, except that in some instances the antisera were placed in the center hole, but in other tests they were situated in the outer wells. At the end of the incubation period, the precipitin bands produced were recorded photographically by dark field illumination, and by drawings.

A sonicated arthrospore and a sonicated spherule agar-gel double diffusion antigen were prepared by sonication for thirty minutes of a 1-20 v/v suspension of arthrospores, and of a 1-20 v/v suspension of a spherule. The supernatant material was clarified in the Model RC2 Refrigerated Servall Centrifuge at 10,500 xG for fifteen minutes.

An autoclaved agar-gel diffusion antigen was prepared from

a 1-20 v/v suspension of washed arthrospores and washed spherules in Seibert's buffer. These two mixtures were autoclaved for fifteen minutes at 120 lbs. pressure at a temperature of 100° C. The supernatant material was then clarified by centrifugation in the refrigerated Servall at 10,500 X G for 15 minutes.

The antisera utilized in this assay was obtained from rabbits infected with C. immitis, and rabbits immunized with formalin-killed spherule, arthrospore, young mycelium, B. dermatitidis, H. capsulatum, and C. albicans cells.

3. Indirect Immuno-Fluorescence. One drop of antigen, delivered with a Pasteur pipette, was placed on alcohol washed, acetone dried microscope slides. Cellular antigens were air dried, and then heat fixed, whereas the sonicated antigens were fixed by immersing the smear in a fifteen per cent acetone solution for ten minutes. The acetone was removed by two successive washings in phosphate buffer (pH 7.2). Two drops of either undiluted immune rabbit sera or undiluted normal rabbit sera were placed over the smear, and the slide was placed in a moist chamber for thirty minutes. The antisera was then removed by washing the slide in two successive changes of phosphate buffer for ten minutes each. After the final washing, two drops of fluorescein isothiocyanate labeled rabbit antiserum globulin (Colorado Serum Co.) were placed over the antigen, and the slide was put back into the moist chamber for thirty minutes. At the end of this incubation period the fluorescein lab labeled antiglobulin was removed by four successive washings of five minutes each in phosphate buffer. The slides were then shaken several times to remove the excess clinging liquid and one drop of HFM immuno-fluorescent mounting fluid (Hartman-Leddon Co., Philadelphia,

Pa.) was placed over the smear. A coverslip was added and the slides were read microscopically on a Leitz Ortholux microscope equipped with a 200 Watt mercury lamp, and a two mm. UG-1 ultraviolet transmittance filter. The U.V. absorbant filter was used in the ocular and the dark field condenser in the microscope.

Photographs were taken with a Leitz micro-attachment camera on Kodak tri-x, 35 mm. film, at an exposure time of five and one half minutes.

An arthrospore immuno-fluorescent antigen was prepared by washing arthrospores three times in physiological saline at 2000 x G in the Model SBV International Centrifuge. After the last centrifugation, the arthrospores were suspended in 0.85 per cent saline at a concentration of approximately one per cent.

A spherule immuno-fluorescent antigen was prepared in the same manner as that described above for arthrospores.

Undiluted anti-arthrospore and undiluted anti-purified spherule pooled sera were used to overlay the antigens in this immuno-fluorescent assay. Undiluted normal rabbit pooled sera was used as the control.

C. Results:

1. Complement fixation tests with pooled sera from infected rabbits.

Cellular antigens. When the pooled sera from infected rabbits was assayed with 1.5 mg/ml of arthrospore, spherule, young mycelium, H. capsulatum, B. dermatitidis, and C. albicans cells, antibody titers of 80, 20, 30, 10, 20, and 20^{+}, respectively, were observed (Table 1).

Twelve mg/ml suspensions of the above mentioned antigens exhibited the following titers in tests with this pooled antisera: 1/32, 1/16, 1/16, 1/8, 1/16, and 1/32, respectively (Table 1).

Sonicated Antigens. When the pooled antisera from infected rabbits was tested with the most reactive dilution of either the arthrospore sonicated, arthrospore sonicated supernatant, or arthrospore sonicated sediment antigens, an antibody titer of 80 was observed. The above antigens exhibited titers of 1/128, 1/64, and 1/32 respectively, in tests with pooled sera from rabbits with coccidioidomycosis (Table 1).

If this antisera was assayed with an optimal dilution of the spherule sonicated, spherule sonicated supernatant, and spherule sonicated sediment antigens, antibody titers of 320, 320, and 160, respectively, were obtained. The above antigens displayed titers of 1/256, 1/256, and 1/16, respectively, in assays with the pooled sera from infected rabbits (Table 1).

Autoclaved-Sonicated antigens. When the most reactive dilution of either the autoclaved-sonicated arthrospore, autoclaved-sonicated arthrospore supernatant, or the autoclaved-sonicated arthrospore sediment antigen was tested with this antisera, an antibody titer of 80 was observed. These antigens displayed titers of 1/256, 1/32, and 1/64, respectively, in tests with the pooled antisera from rabbits with coccidioidomycosis (Table 1).

When optimally reactive dilutions of the autoclaved-sonicated spherule, autoclaved-sonicated spherule supernatant, and autoclaved-sonicated spherule sediment antigens were tested with the pooled antisera from infected rabbits, antibody titers of 320, 160, and 160, respectively, were observed. These antigens exhibited titers of 1/256, 1/128, and 1/64, respectively in assays with this antisera (Table 1).

TABLE 1

Tests with Pooled Sera from Infected Rabbits

Cellular Antigens	Antigen Conc. (mg/ml)	Complement Fixing Antigen Dilution									
		1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Arthrospore cells	12	80	40	40±	neg						
Spherule cells	12	20	10	neg							
Young mycelium cells	12	80	40	neg							
H. capsulatum cells	12	10	neg								
B. dermatitidis cells	12	20	10	neg							
C. albicans cells	12	20±	20±	10	neg						
Sonicated Antigens											
Arthrospore	12	80	80	80±	40	10±	neg.				
Arthrospore supernatant	--	80	80±	40	20	neg.					
Arthrospore sediment	--	80	80±	20	neg.						
Spherule	12	AC	320	320±	160	160	80	neg.			
Spherule Supernatant	--	AC	320	160	160	80	20	neg.			
Spherule Sediment	--	160	80	neg.							

Table 1
(cont)

Tests with Pooled Sera from Infected Rabbits

Autoclaved-Sonicated Antigens	Antigen Conc. (mg/ml)	Complement Fixing Antigen Dilution									
		1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Arthrospore	12	80	80	80	80±	40	10±	neg.			
Arthrospore supernatant	--	80	40	40±	neg.						
Arthrospore sediment	--	80	80	80±	40	neg.					
Spherule	12	AC	320	160	160±	80	20	neg.			
Spherule Supernatant	--	AC	160	160±	80	40	neg.				
Spherule sediment	--	160	160±	80	40	neg.					

2. Agar-gel Double Diffusion. The controls utilized in this assay included Seibert's buffer in the center well, and the various antisera in the outer wells. The different antigens were also tested with normal rabbit sera. All controls gave negative results.

Different dilutions of the sonicated and autoclaved spherule extracts were reacted with varying concentrations of the two anti-spherule sera. Similarly the several soluble arthrospore antigens were migrated toward varying dilutions of the arthrospore antisera. It was observed that all of the antigen-antibody combinations tested formed the most intense and the largest number of precipitin bands when both reactants were undiluted.

It will be seen in Figure 1 that the sonicated spherule antigen formed four precipitin bands in combination with purified spherule antisera. The band closest to this antigen well was very faint, and was possibly not observed in any of the other preparations. The next band appeared as a very thick line. The autoclaved spherule and soluble arthrospore antigens displayed a band in the same position, but it was not as intense. A third band appeared midway between the antigen and antisera wells, and was observed for all of the extracts.

Directly adjacent to the antisera well, two very fine bands were observed. One of these bands appeared to be common to the sonicated arthrospore and spherule preparations, and the other was observed with all solutions except the sonicated spherule.

When the autoclaved and sonicated spherule and arthrospore extracts were migrated toward spherule pooled antisera (Figure 2), two precipitin bands were observed close to the spherule sonicated antigen well. This soluble preparation formed a third band, midway between the antigen and antisera wells, which appeared to be

related to several bands formed by each adjacent extract. One precipitin band observed close to the antisera well appeared to be common to all of the soluble preparations.

When the same extracts were then reacted with arthrospore pooled antisera (Figure 3), it was observed that only the autoclaved and sonicated arthrospore extracts formed one common band in tests with this antisera. The spherule antigens failed to react.

When a pooled antiserum obtained from rabbits with coccidioidomycosis was placed in the center well (Figure 4), all of the soluble antigens formed a thick precipitin band located close to the serum well. These bands merged and did not spur or cross. However, one precipitin band was observed close to the spherule sonicated antigen well which was not seen in any of the other preparations.

The sonicated spherule antigen was then placed in the center well and different immune antisera were situated in the outer wells (Figure 5). This photograph revealed that this antigen formed several precipitin bands with the purified spherule pooled antisera, but did not react with any of the other antisera.

Figure 6 shows the autoclaved spherule antigen in the center well, and the various immune antisera in the outer wells. It was again observed that this preparation formed precipitin bands only in combination with purified spherule pooled antisera.

However, when the sonicated arthrospore antigen was situated in the center well (Figure 7), it formed precipitin bands in combination with purified spherule, arthrospore, and H. capsulatum pooled antisera. This antigen formed several precipitin bands with the purified spherule antisera, and one of these bands

appeared to be identical with the band produced by this antigen with the other two antisera.

Figure 8 shows the autoclaved arthrospore antigen in the center well, and the various immune antisera in the outer wells. This antigen also formed precipitin bands with purified spherule, arthrospore, and H. capsulatum pooled antisera. The bands produced by the autoclaved arthrospore antigen with arthrospore and H. capsulatum pooled antisera appeared to be identical.

The spherule culture supernatant antigen was located in the center well, and the antisera in the outer wells in Figure 9. It was observed that this antigen formed at least five precipitin bands with either purified spherule or spherule pooled antisera. Some of these bands appeared to be identical; others did not. This antigen formed one precipitin band with antisera obtained from rabbits with coccidioidomycosis, and did not react with anti-arthrospore pooled sera.

Figure 10 shows that coccidioidin, placed in the center well, formed two precipitin bands in combination with several different spherule pooled antisera, arthrospore pooled antisera, and pooled antisera obtained from rabbits with coccidioidomycosis.

3. Indirect Immuno-fluorescence.

Cellular antigens. When either undiluted arthrospore or purified spherule pooled antisera were reacted with arthrospore cells, a yellowish green fluorescence at the edge of the cell was observed. This bright staining reaction appeared to involve all or part of the cell wall structure. When the arthrospore cells were tested with undiluted normal rabbit pooled sera, however, only a dull green color appeared.

In the indirect immuno-fluorescent tests in which undiluted anti-arthrospore or anti-purified spherule pooled sera was reacted with the spherule cellular antigen, a definite yellowish-green fluorescent staining of the outer portion of the cell wall of either the spherule or the endospore was observed. This fluorescence tended to outline the stained cells.

D.

Although it was noted that the body of the spherule cells showed some auto-fluorescence, when undiluted normal rabbit pooled sera was tested with these cells, no yellowish green fluorescence at the edge of the spherules or endospores was observed.

Sonicated sediment antigens. The arthrospore sonicated sediment consisted of some apparently intact arthrospores, some very small pieces of material, and some large aggregates of cellular debris. When undiluted anti-arthrospore or anti-purified spherule pooled sera was reacted with this arthrospore sonicated sediment, a "ringing" of the outer portion of the apparently intact arthrospores was observed. The smaller pieces of cellular debris were seen to fluoresce and the large aggregates fluoresced both at the edge and along the surface. When the arthrospore sonicated sediment was tested with undiluted normal rabbit pooled sera, however, no fluorescence was observed.

The spherule sonicated sediment was composed of many small bits of material, frequent large aggregates of cellular debris, and a rare, round endospore-like body. When this antigen was reacted with either undiluted anti-arthrospore or undiluted antipurified spherule pooled sera, the small bits of material emitted pin points of light. The large aggregates fluoresced at the edge and on the surface. The round bodies also demonstrated a positive reaction.

When the spherule sonicated sediment was tested with undiluted normal rabbit pooled sera, some auto-fluorescence was observed. However, this staining reaction was not as bright as when the immune antisera were reacted with this antigen.

D. Discussion:

1. Complement fixation. From the results obtained in tests which utilized pooled sera from infected rabbits and cellular and sonicated antigens, it was observed that sonication increased the antigen titer of the arthrospore and spherule preparations.

It was also observed that this antisera exhibited an antibody titer of 80 regardless of whether it was tested with 1.5 mg/ml of the cellular or sonicated arthrospore antigens. The pooled sera from infected rabbits, however, displayed an antibody titer of 320 when tested with an optimal dilution of the spherule sonicate, and a titer of 20 when tested with an optimal dilution of spherule cells. From these results it was concluded that sonication of arthrospores and spherules made available additional antigenic material for serological reactivity. It was also concluded that the determinants within the arthrospore were similar to those on the spherule surface.

These results were analagous to those obtained with these antigens and immune anti-C. immitis rabbit sera.

2. Agar-gel Double Diffusion. The results of the agar-gel studies were generally analagous to the data obtained by the complement fixation method, and reported in this tri-annual report and the preceding one. Sonicated and autoclaved soluble antigens derived from arthrospores and spherules exhibited at least several precipitin bands when migrated toward purified spherule (Figure 1) and spherule pooled antisera (Figure 2). However, only a single band was formed

when soluble antigens were tested against arthrospore pooled antisera (Figure 3). Again, these results showed that the spherule antigen elicited greater antibody response in the immunized rabbit. Conceivably one or more of these antibodies (Figures 1 and 2) detected in sera derived from rabbits immunized with spherule antigens that were lacking (Figure 3) in sera derived from rabbits immunized with arthrospore antigens might be responsible for the protection of animals immunized with the former antigen (Vogel, et al. 1954; Levine, et al. 1950; Levine, et al. 1962).

Since Huppert and Bailey (1963) showed that human sera obtained from patients with coccidioidomycosis exhibited a minimum of one precipitin band when tested with a soluble antigen from mycelium, it was deemed pertinent to react the sera from infected rabbits with the various soluble antigens (Figure 4). It was noted that all antigens formed one common precipitin band in tests with this sera. An additional precipitin band, however, was elicited by the sonicated spherule antigen in the presence of this sera. This additional band would again emphasize the importance of spherule versus arthrospore antigens in eliciting antibody formation which might account for the protective ability of spherule materials.

Whereas specific antisera were tested by the double diffusion technique against a variety of antigens in Figures 1, 2, 3, and 4, a specific antigen was tested by the double diffusion technique against a variety of antisera in Figures 5, 6, 7, and 8. In these latter Figures it can be seen in 9 that a precipitin band was produced only between sonicated spherule antigen and purified spherule pooled antisera. The other sera tested against this antigen failed to react. This was in agreement with the

reactions of this antigen and these sera in a complement fixation test, with the exception of sera 3 and 4 in Figure 9. Complement fixation tests with these sera (young mycelium and H. capsulatum, preceding tri-annual report) were of sufficient titer, 320, to have expected precipitin bands in a double diffusion test. That these did not occur may be due to a lack of sensitivity in the latter test (Finger and Heller, 1960).

On the other hand, the results of the double diffusion tests with autoclaved spherule antigen versus a variety of antisera (Figure 6) were in complete agreement with the results obtained with this antigen and the same sera in the complement fixation test (preceding tri-annual report). The level of antibody titers in these tests, when they occurred, were of such low magnitude as to have prevented a reaction in a less sensitive assay. Furthermore, it can be seen in Figures 5 and 6 that the spherule antigens were specific in that they did not react with anti-H. capsulatum, anti-B. dermatitids or anti-C. albicans sera in the double diffusion test.

The results of the double diffusion tests with sonicated arthrospore and autoclaved arthrospore antigens tested against a variety of antisera were in agreement with those obtained in complement fixation tests with similar reagents (last two tri-annual reports); bands were produced against anti-purified spherule, anti-arthrospore, and anti-H. capsulatum sera. These antisera exhibited complement fixation titers of 640, 1280, and 320, respectively, when tested with the sonicated arthrospore supernatant antigen, and titers of 320, 640, and 320, respectively, in tests with the autoclaved arthrospore supernatant antigen. The precipitin band produced by the arthrospore extracts in combination with anti-H. capsulatum immunized sera were of particular interest because Heiner (1958) had reported

that a soluble antigen derived from the mycelial phase of C. immitis (coccidioidin) produced a single agar-gel double diffusion precipitin band in tests with serum obtained from patients with histoplasmosis.

It can be said, therefore, that the arthrospore antigens have reacted not only with their homologous sera (arthrospore pooled antisera) but also with heterologous sera (purified spherule and H. capsulatum pooled antisera.) This lack of specificity, particularly with respect to the anti-H. capsulatum sera, did not occur with the spherule antigen discussed above. Moreover, these experiments showed (Figures 5 and 6; 7 and 8) that the arthrospore antigen produced a precipitin band with anti-purified spherule sera whereas the spherule antigen did not react with anti-arthrospore sera. Therefore, it must be assumed that these two antigens differed, quantitatively or qualitively, in their antigenic composition.

Two other antigens (spherule culture supernatant antigen and coccoidioidin) were also tested by the agar-gel double diffusion technique against a variety of sera (Figures 9 and 10). It can be seen in these Figures that spherule antigen again failed to react with arthrospore pooled antisera whereas coccidioidin produced two precipitin bands with this sera. Since coccidioidin was derived from mycelium as was the arthrospore pooled antisera, these results might again indicate quantitative or qualitative differences in antigenic determinants between the spherule and mycelial phases of C. immitis. In Figures 9 and 10 it should also be noted that multiple bands occurred between coccidioidin and test sera whereas the spherule culture supernatant antigen produced multiple bands only with spherule antisera. These results

were also looked upon as indicators of antigenic dissimilarities between the mycelial and spherule growth phases of C. immitis.

3. Indirect Immuno-fluorescence. Vogel and Padula (1958) showed that the cell walls of pathogenic fungi would fluoresce when tested by the indirect immuno-fluorescent technique. Breslau (1957) stated that the polysaccharides in the cell wall of the spherule were the main antigenic agents for stimulating the production of antibodies in disease. Kong, et al. (1963) studied the immunogenicity of disrupted fractions of spherules. They found that the cell wall conferred more protection in mice against challenge with viable C. immitis that did the protoplasmic moiety.

Indirect immuno-fluorescent tests revealed that the walls of either arthrospore or spherule cells or their respective sonicated sediments fluoresced when these antigens were overlayed with either undiluted arthrospore or undiluted purified spherule pooled antisera. It was postulated, therefore, that this fluorescence was another indicator of the antigenicity of the arthrospore and spherule cell walls. These tests possibly revealed the sites of antigenic determinants which played a prominent role in immunity to C. immitis.

E. Future Work

1. A micro-complement fixation assay is now being used to assay arthrospore and spherule antigens, derived by chemical fractionation, against the anti-arthrospore and antispherule sera. Reports in the literature have indicated that this micro-assay is an extremely sensitive complement fixation test.

2. The most antigenic fractions will be tested for their ability to protect animals against challenge with viable C. immitis.

3. An anti-spherule sonicated supernatant and an anti-spherule sonicated sediment serum is now being prepared in rabbits. It is

felt that the use of these sera in the micro-C.F. assay will more readily enable us to identify the protective determinants present in C. immitis.

BIOCHEMICAL DATA

The existence of chitin concentrated in the residue fraction, R6R II, was shown in the previous report. In the present report, the chemical composition of some soluble fractions and the occurrence of a new sugar, 3-O-methylmannose, is reported.

1. Fractionation. Fractionation of the fungal material was performed by the method reported previously. Each soluble fraction was neutralized, dialyzed and evaporated to dryness. Total lipid extracted with chloroform-methanol (2:1) was about 20%, and the phenol extract fraction was about 5% of the dried whole fungus. R6 was further fractionated and the yield of each fraction is shown in Table 1.

The capsule was isolated by differential centrifugation after treatment in the Ribi cell fractionator.

Table 1. Yield of fractions from R6.

Source \ Fraction	$S\,Il_1$	$S\,Il_2$	$S\,Il_3$	RIl	Recovery
Arthrospore	7.2%	26.6%	17.9%	25.5%	79.0%
Mycelium	14.6%	43.5	5.0	16.1	82.2

SIl_1: 1 N HCl extract. SIl_2: conc. Formic acid extract

SIl_3: 10% NaOH extract. RIl: Residue.

2. Chemical composition of fractions. The phenol extract was composed mainly of protein, and the sugar content was less than 1%. Using the paper chromatography and the thin layer

chromatography techniques, seven amino acids were detected by spraying with ninhydrin reagent. These were leucine (or isoleucine), valine, alanine, threonine, serine and a small amount of glycine.

The fraction extracted with concentrated formic acid contained a large amount of carbohydrate, especially glucosamine, and a lesser amount of protein. Mannose and glucose were found as the principal components of the neutral sugar by chromatography, and galactose was demonstrated as the lesser component by using galactose oxidase (Galactostat). A rapidly moving faint spot was also found on the chromatogram. Identification of this sugar will be shown later.

The fraction extracted with 10% sodium hydroxide contained a larger amount of protein and a lesser amount of sugar than the former fraction. Qualitatively, the sugar and amino acid components were not different from the former fraction. In this fraction glucose was weaker and mannose was the dominant neutral sugar as opposed to the glucose of the former fraction.

The capsule fraction contained a higher concentration of glucosamine than did the whole cell. This amino sugar must come from a polymer, and this polymer's resistence to mineral acid suggested chitin. These results also suggested that chitin was condensed in the capsule.

3. Identification of unknown sugar as 3-O-methylmannose. This sugar composed only 1% of the whole arthrospore, or 5% of the total neutral carbohydrate. It was impossible to determine its properties by physico-chemical procedure. A small amount of unknown sugar, which was isolated by thin layer chromatography was applied for identification chromatographically. The color developed

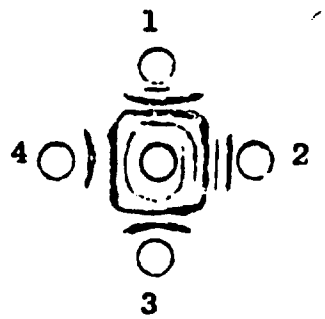
on the chromatogram by spraying with p-anisidine, aniline hydrogen phthalate, and 2,3, 5-triphenyltetrazolium chloride reagent showed that this sugar was an aldohexose derivative and that the C2 of the hexose was not substituted with a methoxyl group. R_f values determined on paper and thin layer with four different solvent systems corresponded with known 3-O-methylmannose.

4. Lipids in arthrospores. A twenty per cent suspension of whole arthrospores was extracted with a mixture of chloroform; methanol (2:1). The extracted lipid was fractionated into seven fractions on a silicic acid column. These fractions, however, were not homogenous by thin layer chromotography. The components of these lipid fractions are as yet unidentified.

Total lipid was saponified with methanolic KOH and the fatty acids separated. These are under analysis with gas liquid chromotography.

FIGURE 1

AGAR-GEL DOUBLE DIFFUSION TEST



Center well:

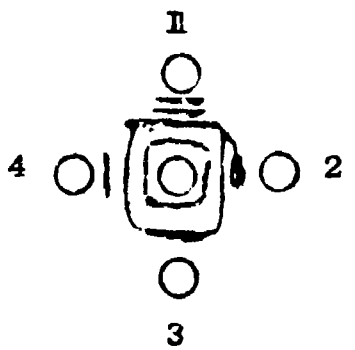
Purified spherule pooled antisera

Outside Wells:

1. Sonicated spherule antigen
2. Sonicated arthrospore antigen
3. Autoclaved arthrospore antigen
4. Autoclaved spherule antigen

FIGURE 2

AGAR-GEL DOUBLE DIFFUSION TEST

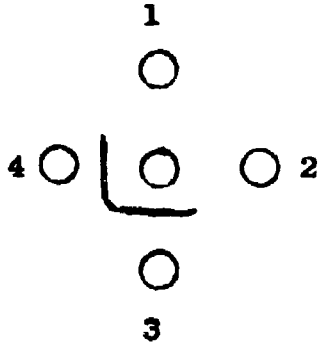


Center well:
Spherule pooled antisera

Outside wells:
1. Sonicated spherule antigen
2. Autoclaved spherule antigen
3. Autoclaved arthrospore antigen
4. Sonicated arthrospore antigen

FIGURE 3

AGAR-GEL DOUBLE DIFFUSION TEST

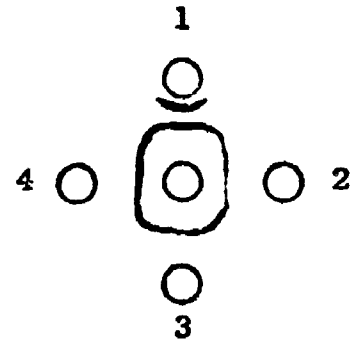


Center Well:
Arthrospore pooled antisera

Outside Wells:
1. Sonicated spherule antigen
2. Autoclaved spherule antigen
3. Sonicated arthrospore antigen
4. Autoclaved arthrospore antigen

FIGURE 4

AGAR-GEL DOUBLE DIFFUSION TEST

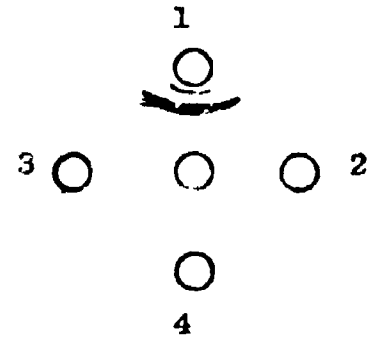


Center Well:
Pooled antisera from rabbits with coccidioidomycosis

Outside Wells:
1. Sonicated spherule antigen
2. Autoclaved spherule antigen
3. Sonicated arthrospore antigen
4. Autoclaved arthrospore antigen

FIGURE 5

AGAR-GEL DOUBLE DIFFUSION TEST

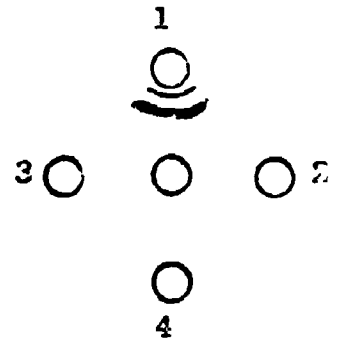


Center Well:
Sonicated spherule antigen

Outside Wells:
1. Purified spherule pooled antisera
2. Arthrospore pooled antisera
3. Young mycelium antisera
4. H. capsulatum pooled antisera

FIGURE 5
(cont.)

AGAR-GEL DOUBLE DIFFUSION TEST

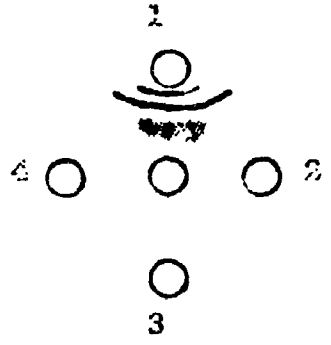


Center Well:
Sonicated spherule antigen

Outside wells:
1. Purified spherule pooled antisera
2. Arthrospore pooled antisera
3. C. albicans antiserum
4. B. dermatitidis antiserum

FIGURE 3

AGAR-GEL DOUBLE DIFFUSION TEST

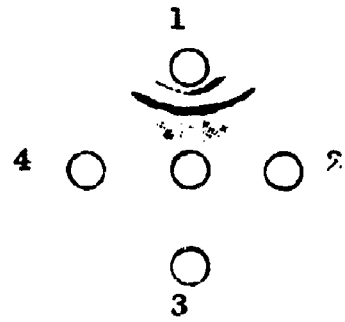


Center well:
Autoclaved spherule antigen

Outside wells:
1. Purified spherule pooled antisera
2. Arthrospore pooled antisera
3. Young mycelium antiserum
4. H. capsulatum pooled antisera

FIGURE 3
(cont.)

AGAR-GEL DOUBLE DIFFUSION TEST

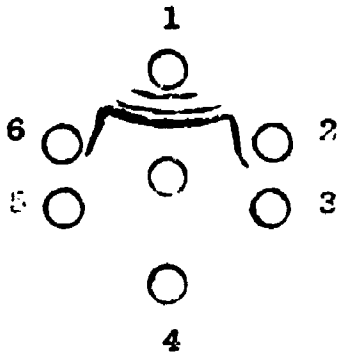


Center well:
Autoclaved spherule antigen

Outside wells:
1. Purified spherule pooled antisera
2. Arthrospore pooled antisera
3. C. albicans antiserum
4. B. dermatitidis antiserum

FIGURE 7

AGAR-GEL DOUBLE DIFFUSION TEST

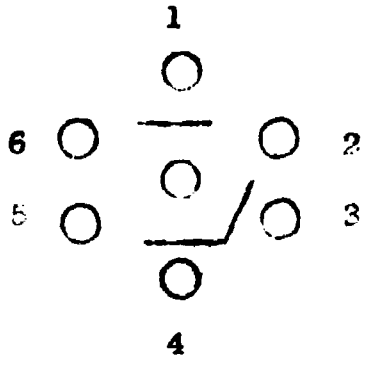


Center well:
Sonicated arthrospore antigen

Outside wells:
1. Purified spherule pooled antisera
2. Arthrospore pooled antisera
3. C. albicans antiserum
4. B. dermatitidis antiserum
5. Young mycelium antiserum
6. H. capsulatum pooled antisera

FIGURE 8

AGAR-GEL DOUBLE DIFFUSION TEST

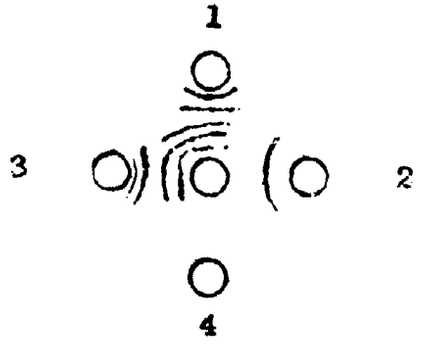


Center well:
Autoclaved arthrospore antigen

Outside wells:
1. Purified spherule pooled antisera
2. C. albicans antiserum
3. Arthrospore pooled antisera
4. H. capsulatum pooled antisera
5. B. dermatitidis antiserum
6. Young mycelium antiserum

FIGURE 9

AGAR-GEL DOUBLE DIFFUSION TEST

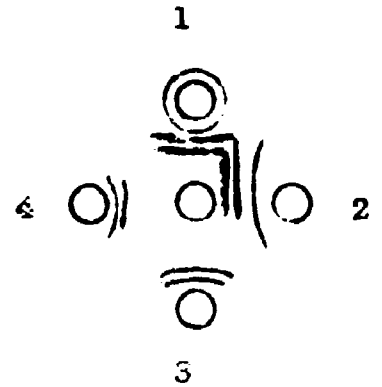


Center well:
Spherule culture supernatant antigen

Outside wells:
1. Purified spherule pooled antisera
2. Pooled antisera obtained from rabbits with coccidioidomycosis
3. Arthrospore pooled antisera
4. Spherule pooled antisera

FIGURE 10

AGAR-GEL DOUBLE DIFFUSION TEST



Center well:
Coccidioidin

Outside wells:
1. Purified spherule pooled antisera
2. Pooled antisera obtained from rabbits with coccidioidomycosis
3. Arthrospore pooled antisera
4. Spherule pooled antisera

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